Lipase-Catalyzed Methanolysis of Triricinolein in Organic Solvent to Produce 1,2(2,3)-Diricinolein

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ABSTRACT: The objective of this study was to find the optimal parameters for lipase-catalyzed methanolysis of triricinolein to produce 1,2(2,3)-diricinolein. Four different immobilized lipases were tested, *Candida antarctica* type B (CALB), *Rhizomucor miehei* (RML), *Pseudomonas cepacia* (PCL), and *Penicillium roquefortii* (PRL). *n*-Hexane and diisopropyl ether (DIPE) were examined as reaction media at three different water activities (a_w), 0.11, 0.53, and 0.97. The consumption of triricinolein and the formation of 1,2(2,3)-diricinolein, methyl ricinoleate, and ricinoleic acid were followed for up to 48 h. PRL gave the highest yield of 1,2(2,3)-diricinolein. Moreover, this lipase showed the highest specificity for the studied reaction, i.e., high selectivity for the reaction with triricinolein but low for 1,2(2,3)-diricinolein. Recoveries of 93 and 88% DAG were obtained using PRL in DIPE at a_w of 0.11 and 0.53, respectively. Further, NMR studies showed that a higher purity of the 1,2(2,3)-isomer vs. the 1,3-isomer was achieved at higher a_w (88% at a_w = 0.53), compared to lower a_w (71% at a_w = 0.11). The DAG obtained was acylated by the DAG acyltransferase from *Arabidopsis thaliana*. Therefore, this enzymatic product is a useful enzyme substrate for lipid biosynthesis. Accordingly, the use of PRL in DIPE at a_w 0.53 is considered optimal for the synthesis of 1,2(2,3)-diricinolein from triricinolein.

Paper no. L9376 in *Lipids 38*, 1197–1206 (November 2003).

The final step in the biosynthetic pathway of oil in castor seeds (*Ricinus communis* L.) is the acylation of 1,2-diricinolein to triricinolein (1). Triricinolein makes up the major part of the oil in castor seed, around 71% (2). The oil is highly viscous and is used industrially in products such as lubricants, coatings, polymers, and cosmetics (3). Unfortunately, castor seeds contain a potent toxin, ricin, which inhibits protein synthesis in mammalian cells by attacking the ribosome (4). In addition, they contain an allergen; therefore, work has been done in our laboratory to produce triricinolein in a plant lacking toxins. One important step has been to clone the cDNA for DAG acyltransferase (DGAT) in castor seeds, one of the enzymes involved in the biosynthesis of triricinolein from 1,2-diricinolein. To characterize the specific activity of the expressed DGAT enzyme, 1,2-diricinolein was needed as a substrate. Hence, the aim of this work was to synthesize a significant amount of 1,2-diricinolein.

There is also a more general interest in the synthesis of DAG, since they are useful as emulsifiers in food, pharmaceuticals, and cosmetics. DAG oil is a useful substitute for TAG oil in food (5). Furthermore, since 1,2-DAG can be used as starting material for the synthesis of various prodrugs (6), it is important to use an isomerically pure compound.

1,2-DAG are difficult to synthesize by using conventional organic methodologies. Instead, lipases can be used for higher selectivity of the reaction. Whichever method is used, enzymatic or chemical, the DAG formed are always a mixture of 1,2-, 2,3-, and 1,3-isomers, although the proportions vary. The optical isomers, 1,2- and 2,3-DAG, are usually referred to as 1,2(2,3)-DAG, since they are difficult to separate using common chromatography systems. For example, Fureby *et al.* (7) showed that lipases could be used for selective preparation of 1,2(2,3)-DAG from TAG by alcoholysis in organic media. In that work, 22 different lipases were tested for activity toward trilaurin, tricaprin, and tripalmitin, and a lipase from *Penicillium roquefortii* gave the highest yield of 1,2(2,3)-DAG. It is possible to produce 1,2-DAG from acylation (chemical or enzymatic) of enzymatically produced glycerol-3-phosphate followed by dephosphorylation (8). This approach has been used for chemical synthesis of labeled DAG. However, the presence of a hydroxyl group on ricinoleate makes chemical acylation difficult.

In this work, lipases were used to catalyze the hydrolysis and methanolysis of triricinolein to 1,2(2,3)-diricinolein. This approach is rather different from the one described above (7), since the presence of a hydroxyl group on the hydrocarbon chain probably alters the interaction of a lipase with triricinolein compared to saturated TAG. Further, there is a potential for polymerization of liberated ricinoleic acid, forming estolides, which also may affect the reaction.

Four different immobilized lipases were studied—Novozyme 435 *(Candida antarctica* type B, CALB), Lipozyme RM-IM *(Rhizomucor miehei,* RML), Lipase PS *(Pseudomonas cepacia*, PCL), and Lipase R_G *(P. roquefortii,* PRL). The reactions were performed in two different solvents, *n*-hexane and diisopropyl ether (DIPE)—at three water activities (a_w), 0.11, 0.53, and 0.97. 1,2(2,3)-Diricinolein was identified by MS, and its purity vs. 1,3-diricinolein was determined by ^{1}H and

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Abbreviations: AtDGAT, cDNA coding DGAT cloned from *Arabidopsis thaliana*, Columbia; a_w, water activity; CALB, *Candida antarctica* type B lipase; DGAT, DAG acyltransferase; DIPE, diisopropyl ether; ESI-MS, electrospray ionization MS; NP-HPLC, normal-phase HPLC; PCL, *Pseudomonas cepacia* lipase; PRL, *Penicillium roquefortii* lipase; RML, *Rhizomucor miehei* lipase; RRO, *sn*-1,2-diricinoleoyl-*sn*-3-oleoyl TAG; RT-PCR, reverse-transcriptase PCR.

^{13}C NMR. The conditions giving the highest yield of diricinolein were selected for synthesis.

EXPERIMENTAL PROCEDURES

HPLC system. A Waters Millenium HPLC system consisting of a computer, a gradient pump (model 600), an autosampler (model 717), and a UV-vis detector (model 2487) (Waters Associates, Milford, MA) was used. A Beckman Ultrasphere column, ODS 5 μm, 250 × 4.6 mm, was used for analysis (Beckman Instruments Inc., Fullerton, CA); a Phenomenex Luna column, C18(2) 100 Å, 5 μm, 250 × 15 mm (Phenomenex, Torrance, CA) was used for preparative chromatography; and a Phenomenex Luna column, CN 5 μm, 250 × 4.6 mm, was used for normal-phase HPLC (NP-HPLC).

Solvents. HPLC-grade 2-propanol, methanol, and hexane were obtained from Fisher Scientific (Fairlawn, NJ), DIPE was purchased from Fluka Chemie (Buchs, Switzerland), and methyl *tert*-butyl ether (MTBE) was obtained from Aldrich (Milwaukee, WI).

Chemicals. Accurel MP1000 was obtained from Membrana (Obernburg, Germany). Lithium chloride and methyl ricinoleate were purchased from Sigma (St. Louis, MO). Sodium sulfate and magnesium nitrate were obtained from J.T.Baker Inc. (Phillipsburg, NJ). Potassium sulfate, sodium phosphate, and disodium phosphate were purchased from Spectrum Chemical Mfg. Corp. (Gardena, CA).

Enzymes. Crude PCL and PRL were kind gifts from Amano Enzymes Inc. (Nagoya, Japan). Novozyme 435 (immobilized CALB, 10.000 propyl laurate units/g) and Lipozyme RM-IM [immobilized RML, 6.1 batch acidolysis units Novo (BAUN)/g] were generous gifts from Novozymes North America (Franklinton, NC). The activity for Novozyme 435 is based on ester synthesis of 1-propanol and lauric acid at 60°C for 15 min. Ester formation is calculated based on the acid values of the reaction mixture measured by titration before and after the reaction, and given as propyl laurate units/g. BAUN is based on acidolysis of high-oleic acid sunflower oil and decanoic acid at 70°C for 60 min. The rate of the reaction is determined by measuring the amount of decanoic acid incorporated into the 1- and 3-positions of the TAG.

Preparation of triricinolein. Triricinolein is not commercially available and was produced in our laboratory by preparative chromatography of castor oil, which is 71% triricinolein. Castor oil (0.5 mL at 0.5 g/mL in 2-propanol) was injected onto the preparative column. The mobile phase used was a gradient of methanol (A) and 2-propanol (B), and the flow rate was 7 mL/min, eluted with 100% A for 5 min, a linear gradient of A/B (80:20) over 10 min, and held for 5 min. The column was then reconditioned back to 100% A over 5 min and held for 5 min. Triricinolein was collected at a retention time of around 10 min. The solvent was removed under nitrogen with mild heating. The purity of the fractions was checked by analytical HPLC.

Immobilization of lipases. PCL and PRL were immobilized on a polypropylene powder (Accurel MP1000, formerly called EP100) by adsorption as described by Gitlesen *et al.* (9). Five grams of crude enzyme was mixed with 100 mL of phosphate buffer (pH 6.0, 20 mM) and then added to 5 g of Accurel MP1000 that had been prewetted with 15 mL of ethanol. The mixture was incubated overnight at room temperature while stirring. The following day the mixture was filtered, and the filtrate was washed with 5 mL of phosphate buffer (pH 7.0, 200 mM). The immobilized enzyme was dried overnight under vacuum.

Equilibration of the reaction system. The immobilized enzymes, reaction media (hexane and DIPE), and methanol were allowed to equilibrate for at least 48 h to a certain a_w over saturated salt solutions (10). This was achieved in airtight desiccators in which saturated water solutions of LiCl, $MgNO_3$, and K_2SO_4 gave a_w of 0.11, 0.53, and 0.97, respectively.

Methanolysis reaction. One hundred milligrams of triricinolein was dissolved in 5 mL of reaction media and 0.5 mL of methanol. One hundred microliters was taken out as a first fraction to determine the starting concentration of triricinolein (which was set to 100% yield, as shown on the axis of Fig. 5 and 6). Thereafter, 100 mg of immobilized enzyme was added, and the reaction took place in rotating glass tubes at room temperature. One hundred-microliter fractions were taken out at time intervals (after 0.5, 1, 2, 4, 6, 24, and 48 h), the solvent of each fraction collected was removed by nitrogen, and 0.5 mL of 2-propanol was added. The fractions were stored in a freezer at −20°C until HPLC analysis. The reactions were performed in duplicate.

HPLC analysis. Expected products of the lipase reaction, i.e., ricinoleic acid, methyl ricinoleate, diricinolein, and triricinolein, were analyzed by injection of 20 μL of sample on an RP-HPLC column, using a mobile phase of methanol (A) and methanol/water (90:10) (C). The gradient was as follows: from 100% C to 100% A in 20 min, then held for 10 min, and a gradient back to 100% C in 5 min, then held for 5 min. The total run time was 40 min. The flow rate was 1.0 mL/min, and the detection was performed at 205 nm. The peaks were quantified using external calibration with methyl ricinoleate at three different concentrations between 0.5 and 5.0 mg/mL.

Optimized methanolysis reaction. Enzymes and solvents were pre-equilibrated to a_w 0.53 for at least 48 h. One hundred milligrams of triricinolein was dissolved in 5 mL of DIPE and 0.5 mL of methanol. One hundred milligrams of PRL was added, and the mixture was allowed to react for 24 h during stirring at room temperature. The enzyme was removed by filtration at the end of the reaction time. The solvent was removed by nitrogen, and one mL of 2-propanol was added.

Fractionated collection of diricinolein. Portions of reaction product from above (0.5 mL) were injected onto the preparative column. A mobile phase consisting of methanol (A) and methanol/water (90:10) (C) was used at a flow rate of 7.0 mL/min. The gradient elution was from 100% C to 100% A in 15 min, then held for 15 min, back to 100% C in 5 min, and held for 5 min. The total run time was 40 min. The diricinolein peak was collected at a retention time of 16 min. Anhydrous sodium sulfate was added to the fractions collected, and the

tubes were shaken for 60 min. The sodium sulfate was removed by filtration, and the solvent was removed by nitrogen under mild heating. The recovered diricinolein oil was stored at –20°C.

MS. MS analysis was performed on an API QStar Pulsar Hybrid Quadrupole-TOF LC/MS/MS mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a Protana nanospray ion source (Proxeon Biosystems, Odense, Denmark). Prior to the electrospray ionization (ESI)-MS experiments, each sample was diluted with acetonitrile/water (1:1) containing 0.1% trifluoroacetic acid. The MS instrument was externally calibrated with Glu-Fib (Sigma). The samples were loaded into individual spray tips made of glass (PicoTip emitters; New Objective Inc., Woburn, MA) and inserted directly into the electrospray interface. The reactions were performed in duplicate and the mean values were reported.

NMR spectroscopy. NMR spectra were obtained at 27°C from samples in $CDCl_3$ with tetramethylsilane as an internal standard on a Bruker model ARX400 spectrometer (Bruker BioSpin Corp., Fremont, CA) at a frequency of 100.62 MHz for carbon and 400.13 MHz for protons. A 30° pulse at a 2.3-s repetition rate was used for carbon, and a 90° pulse at a 7- to 8-s repetition rate was used for protons. The number of attached protons for the ^{13}C signals was determined from distortionless enhancement by polarization transfer (DEPT) 90 and DEPT135 assays.

NP-HPLC. Peak purity of the 1,2(2,3)-isomer vs. the 1,3-isomer of diricinolein was also determined by NP-HPLC. Twenty-microliter samples in DIPE were injected onto a CN column, with a mobile phase consisting of *n*-hexane and MTBE (7:3), isocratic run. The total analysis time was 16 min. The flow rate was 1.0 mL/min with UV detection at 205 nm. The 1,3- and 1,2(2,3)-isomers eluted 2 min apart, at around 11 and 13 min retention time, respectively.

Cloning and expression of a cDNA encoding DGAT from A. thaliana*, Columbia (AtDGAT).* A full-length AtDGAT cDNA was amplified from an RNA sample extracted from *Arabidopsis* leaves by reverse transcriptase PCR (RT-PCR). Specific primers were designed based on the sequence information from GenBank (AF 051849): 5′-GAAATGGCGATTTTGGATTCT-GCT-3′ and 5′-TGACATCGATCCTTTTCGGTTCAT-3′. The cDNA was cloned into a pYES2.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA) and verified by complete sequencing in both directions using a PerkinElmer Big Dye Sequencing Kit (PerkinElmer, Seer Green, Buckinghamshire, United Kingdom). The recombinant protein was expressed in *Saccharomyces cerevisiae* strain INVSc-1 by using the pYES 2.1 TOPO TA Expression Kit according to the manufacturer's instructions (Invitrogen). Briefly, a single colony containing the pYES2.1/V5-His/*AtDGAT* construct was inoculated into medium containing 2% glucose and grown overnight at 30°C with shaking. Galactose (2%) was then added to the medium to induce expression of *AtDGAT* from the *GAL1* promoter. Cells were harvested after induction and the cell pellets were stored at –80°C until ready to use.

Assay for DGAT activity using lipase-catalyzed 1,2(2,3)-diricinolein as a substrate. Microsomes were isolated from harvested yeast cells as described by Urban *et al.* (11) and resuspended in 0.1 M Tris-HCl, pH 7.0, containing 20% glycerol and kept frozen at –80°C. Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). DGAT assays were performed as described in Cases *et al*. (12) with slight modifications. ^{14}C-Labeled oleoyl-CoA was synthesized according to McKeon *et al*. (13). The reaction mixture (100 μL) consisted of 0.1 M Tris-HCl, pH 7.0, containing 20% glycerol, microsomes (50 μg of protein), 1,2(2,3)-diricinolein (1.0 mM), and ^{14}C-oleoyl-CoA (20 μM, 200,000 cpm) and was incubated for 15 min at 30°C. The reactions were stopped and lipids were extracted using chloroform/methanol as described previously (14). Molecular species of acylglycerols were separated on a C18 column (25 × 0.46 cm, 5 μm, Ultrasphere C18; Beckman Instruments Inc.) using HPLC (15). Enzyme activity was determined based on the ^{14}C-label incorporated into the TAG products.

RESULTS AND DISCUSSION

A general reaction path for lipase-catalyzed hydrolysis of TAG is shown in Figure 1A. Lipases usually hydrolyze the outer positions first (*sn*-1 or *sn*-3), as these are more available than the inner *sn*-2 position. Once 2-MAG has been formed, most lipases easily break the last ester bond to form glycerol and FFA. Note that acyl migration occurs to form 1,3-DAG from 1,2(2,3)-DAG and 1(3)-MAG from 2-MAG. This type of migration is especially prominent in protic hydrophilic solvents such as ethanol and methanol and in aprotic hydrophobic solvents such as hexane, but it is lower in dipolar hydrophobic solvents such as ethers and ketones (16).

Figure 1B shows the details of the reaction studied in this work, i.e., the hydrolysis/methanolysis of triricinolein to 1,2(2,3)-diricinolein. The first step of Figure 1B describes the hydrolysis of the ester bond, which results in the release of 1,2(2,3)-diricinolein. The second step—hydration or methylation of the FA—is more rate limiting (17). The aim of this work was to find a lipase that selectively catalyzes this reaction (without further degradation of diricinolein to monoricinolein and glycerol, as described in Fig. 1A). Hence, the lipase should either show very low activity with 1,2(2,3)-diricinolein or give high yields of diricinolein before further degradation is initiated (i.e., much faster reaction kinetics with triricinolein than with diricinolein). Furthermore, acyl migration should be minimized, preferably by performing the reaction in a solvent such as ether.

HPLC analysis. The progress and yield of the reaction were monitored by determining the concentrations of triricinolein consumed and ricinoleic acid, methyl ricinoleate, and 1,2(2,3)-diricinolein formed. Monoricinolein was not found in the fractions, indicating that the last FA of the glycerol backbone was rapidly methanolyzed to give free glycerol and methyl ricinoleate. This effect also has been observed by others (7).

Figure 2 shows a chromatogram of a fraction from the PRL-catalyzed reaction of triricinolein in DIPE at a_w 0.11. The components considered were clearly well separated within 30 min using RP-HPLC. The peak eluting just before methyl ricinoleate, BHT, is a stabilizer in the DIPE solvent.

Diricinolein eluted as one peak in this RP-HPLC system, i.e., as a mixture of 1,2-, 2,3-, and 1,3-isomers. ESI-MS and MS-MS provided supporting evidence for identification of this peak as diricinolein. The ESI-mass spectrum of diricinolein is shown in Figure 3A. The protonated parent ion $(M + H)^+$ had a measured mass of 653.5 Da, which corresponds well to the calculated monoisotopic M.W. of protonated diricinolein, 653.5337 Da. This sample also gave rise to a sodium adduct $(M + Na)^+$ at *m/z* 674.5 and fragments at 652.5 $(M)^+$, 634.5 $(M - H_2O)^+$, 616.5 $(M - 2H_2O)^+$, and 598.5 $(M - 3H_2O)^+$. The parent ion was further fragmented by tandem MS-MS, giving the spectra in Figure 3B. The same ions were found as described above for the single MS mode, in addition to two further fragmented ions of *m/z* 337.3 and 355.3, respectively. The latter of these is the parent ion minus one of the FA chains (see Fig. 3B), and the former is the same but minus a water molecule. In conclusion, the MS experiments verified that it was diricinolein that was formed in the enzymatic reaction experiments.

1,2- and 2,3-Diricinolein are optical isomers (enantiomers), and they are difficult to separate using ordinary HPLC techniques. They may be separated as 3,5-dinitrophenyl urethanes using chiral HPLC (18), but the procedure is rather tedious. However, 1,3-diricinolein is a structural isomer that can be separated more easily from the 1,2(2,3)-isomers. Initially, some effort was made to separate 1,2(2,3)-diricinolein from 1,3-diricinolein using RP-HPLC and solvents such as methanol, 2-propanol, and water as the mobile phase. However, these structural isomers could be partly separated only by using the reversed-phase column. ^1H and ^{13}C NMR were used to determine the purity of 1,2(2,3)-diricinolein, but a less tedious

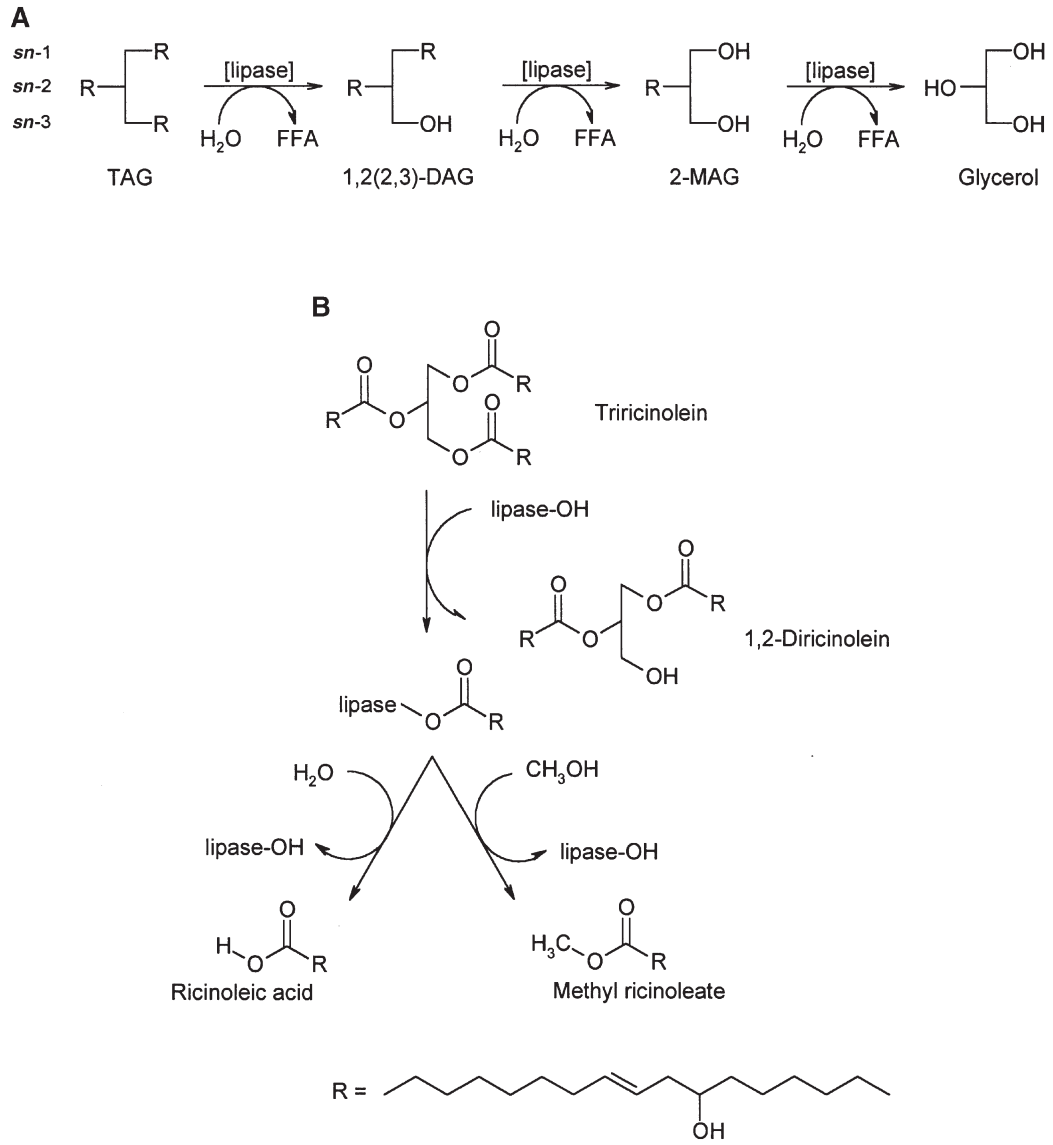

FIG. 1. (A) General reaction scheme for lipase-catalyzed hydrolysis of TAG to glycerol and FFA. (B) Reaction scheme for lipase-catalyzed hydrolysis/methanolysis of triricinolein to 1,2(2,3)-diricinolein and FFA/FAME.

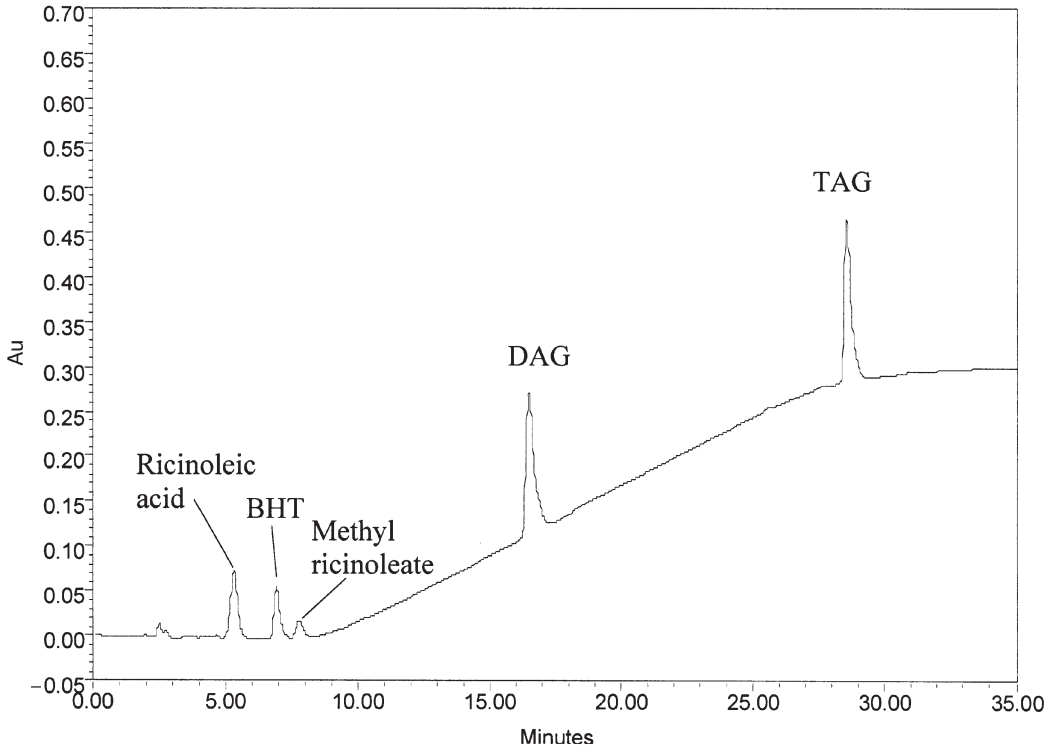

FIG. 2. RP-HPLC analysis of a fraction from the *Penicillium roquefortii* lipase (PRL)-catalyzed reaction in diisopropyl ether (DIPE) at water activity (a_w) of 0.11. An ODS column (Beckman Instruments Inc., Fullerton, CA) was used, and the mobile phase was based on methanol and water (see the Experimental Procedures section). The peaks were identified as ricinoleic acid, BHT, methyl ricinoleate, diricinolein (DAG), and triricinolein (TAG).

NP-HPLC procedure also was developed for the same purpose. Figure 4 shows the excellent separation of 1,3- and 1,2(2,3)-diricinolein isomers. This system was also used for fractionated collection of small amounts of pure 1,2(2,3)-diricinolein.

Choice of lipase. Four different lipases were tested in this study: CALB, RML, PCL, and PRL. In a previous study on ethanolysis of vitamin A esters in DIPE and hexane, CALB, RML, and PCL showed the best activity and stability of the six lipases studied (19). PRL was selected for the present study since it has demonstrated high specificity for conversion of TAG to 1,2(2,3)-DAG (7).

Twenty-four experiments were performed to evaluate the effects of solvents (hexane and DIPE) as well as a_w (0.11, 0.53, and 0.97) on the yield of 1,2(2,3)-diricinolein ($n = 2$). PRL was shown to have a higher specificity for the desired reaction than the other lipases investigated (Fig. 5).

Figure 5A shows the yield of diricinolein in DIPE at a_w 0.11 when CALB was used as the catalyst. The highest yield (40%) of diricinolein occurred after only 1 h of reaction, and it was then rapidly consumed for further production of methyl ricinoleate. RML and PCL showed similar trends. This behavior is common in lipases, as they usually drive the reaction toward degradation of TAG to either glycerol (nonspecific lipases) or 2-MAG (1,3-specific lipases) and FFA or FAME (see Fig. 1A).

Figure 5B shows the yield of diricinolein when PRL catalyzed the reaction in the same solvent and at the same a_w. In this case, the reaction was driven less toward methyl ricinoleate formation and more toward formation of diricinolein. The yield reached a maximum of 93% after around 24 h of reaction and remained stable for at least another 24 h. This result confirms that PRL selectively converts TAG to DAG but does not react further (7).

The maximum yields of diricinolein for all the reaction conditions tested are given in Table 1. PRL was clearly the preferred lipase for the reaction in question, giving diricinolein yields between 63 and 93% for a_w of 0.11 and 0.53 in hexane and DIPE.

Choice of solvent. Hexane and DIPE were investigated as reaction media for this study, since these solvents are commonly used for lipase-catalyzed reactions. In general, solvents of a higher log*P*-value (such as hexane: log*P* ~ 3.9) cause less inactivation of the enzyme than solvents of a low log*P*-value (such as ethanol and acetonitrile: log*P* < 0.2) (20). However, reaction rates are faster in solvents of a lower log*P*-value. Hence, a solvent of an intermediate log*P*-value, such as DIPE (log*P* ~ 1.7), may be a good compromise.

Of the two solvents studied, hexane and DIPE, the latter enabled slightly faster conversion of triricinolein to diricinolein using PRL (see Table 1). In hexane at any a_w, the maximum yield of diricinolein (83%) was not obtained until after 48 h of reaction. Therefore, DIPE was considered the better reaction medium for the reaction catalyzed by PRL. In addition, acyl migration was slower in DIPE than in hexane (16).

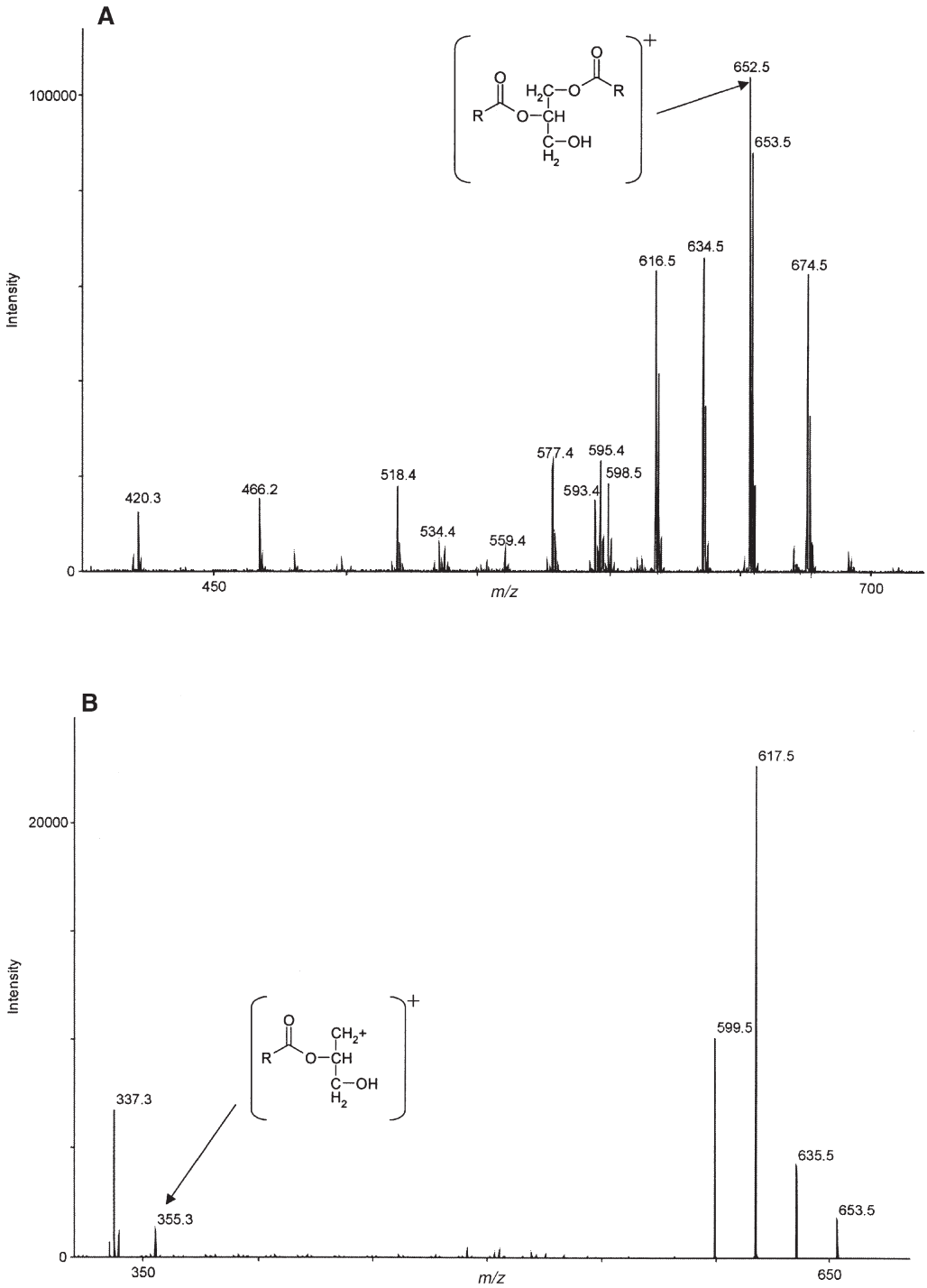

FIG. 3. (A) Mass spectrogram of diricinolein generated in the single MS mode, and the chemical structure of 1,2(2,3)-diricinolein. (B) Mass spectrogram of the parent ion of diricinolein generated in the tandem MS-MS mode, and the chemical structure of 1,2(2,3)-diricinolein minus one of its FA chains. R = ricinoleic acid, as illustrated in Figure 1.

Optimization of a_w. Three different a_w were studied, 0.11, 0.53, and 0.97. In general, a lower a_w gave a higher yield of diricinolein with any of the enzymes in any of the solvents. However, RML and PCL in DIPE produced diricinolein at higher yields with higher a_w (Table 1). This result is in agreement with work demonstrating that the initial reaction rate of PCL in DIPE was faster at higher a_w (21).

The PRL-catalyzed reaction in DIPE at a_w 0.53 and 0.97 is demonstrated in Figures 6A and 6B, respectively. The corresponding reaction at a_w 0.11 is shown in Figure 5B. These

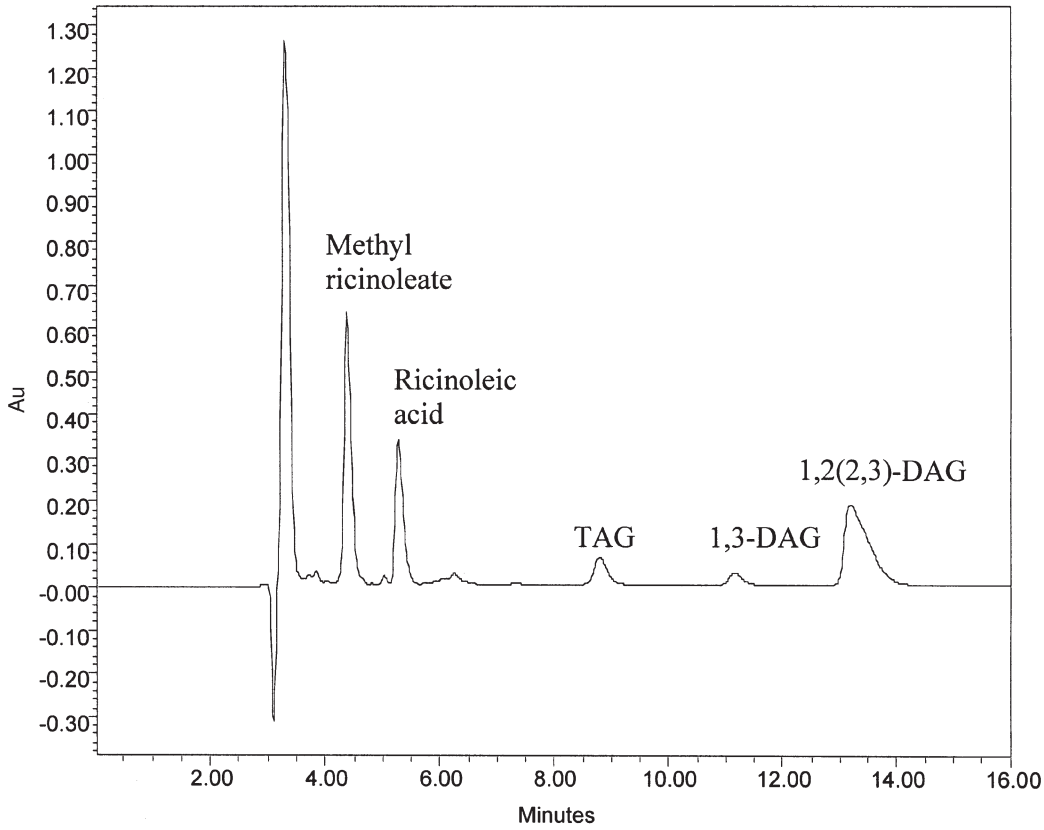

FIG. 4. Normal-phase HPLC analysis of a fraction from the PRL-catalyzed reaction in DIPE at an a_w of 0.53. A CN column (Phenomenex, Torrance, CA) and a mobile phase consisting of hexane and methyl *tert*-butyl ether (see the Experimental Procedures section) were used. The peaks were identified as methyl ricinoleate, ricinoleic acid, triricinolein (TAG), 1,3-diricinolein (1,3-DAG) and 1,2(2,3)-diricinolein [1,2(2,3)-DAG]. For other abbreviations see Figure 2.

figures demonstrate that, within the reaction time studied (48 h), the highest recoveries were obtained at a_w 0.11 and 0.53 (93 and 88%, respectively). At the highest a_w (0.97 in Fig. 6B), the reaction was very slow, and maximum yield was not reached even after 48 h of reaction. Since the reaction yields were similar at a_w 0.11 and 0.53, these two experiments were repeated for further examination.

The purity of 1,2(2,3)-diricinolein vs. 1,3-diricinolein was determined using ^{1}H and ^{13}C NMR. The isomeric structures of the mixture were identified by the chemical shift value of the proton attached to the *sn*-2 carbon of the glycerol backbone. For 1,2(2,3)-diricinolein, this proton was shown as a pentet at 5.080 ppm, whereas 1,3-diricinolein had a multiplet at 4.159 ppm. The observed data are compatible with those reported in the literature for triacetin and tristearin (absorption maxima at 242 and 368, respectively, in Ref. 22). The percentage of the isomeric components present in the mixture was calculated from the comparative integral value of each component in the spectral data of the mixture.

Although a_w 0.11 gave a higher yield of diricinolein, the proportion of 1,2(2,3)-isomer vs. 1,3-isomer was lower, only 73%, compared with that for a_w 0.53, where it was 88%. Therefore, an a_w of 0.53 was selected as optimal for the studied reaction. Additional experiments using a high a_w (~0.97) showed that the reaction took several days to give high yields, although the purity of 1,2(2,3)-diricinolein formed was 93%. Since the 1,2(2,3)-isomer was readily separated from the 1,3-isomer using NP-HPLC, giving a pure product (less than 1% of 1,3-diricinolein), for our purposes there was no point in carrying out the longer reaction to obtain a slight improvement in purity.

The DGAT catalyzes the final step in the Kennedy pathway for TAG biosynthesis and uses acyl-CoA to acylate 1,2-diricinolein. To test whether 1,2(2,3)-diricinolein could serve as an effective substrate for AtDGAT, an AtDGAT cDNA was expressed in yeast *S. cerevisiae* strain INVSc-1 by using a pYES2.1-TOPO TA Expression Kit. Microsomes containing AtDGAT proteins were extracted and used for the DGAT activity assay.

The DGAT activity was 510 pmol/min/mg protein when we supplied the microsomes with 1 mM 1,2(2,3)-diricinolein, whereas the activity was only 330 pmol/min/mg protein without adding diricinolein. The incorporation of ^{14}C-labeled oleoyl-CoA into ^{14}C-labeled *sn*-1,2-diricinoleoyl-*sn*-3-oleoyl

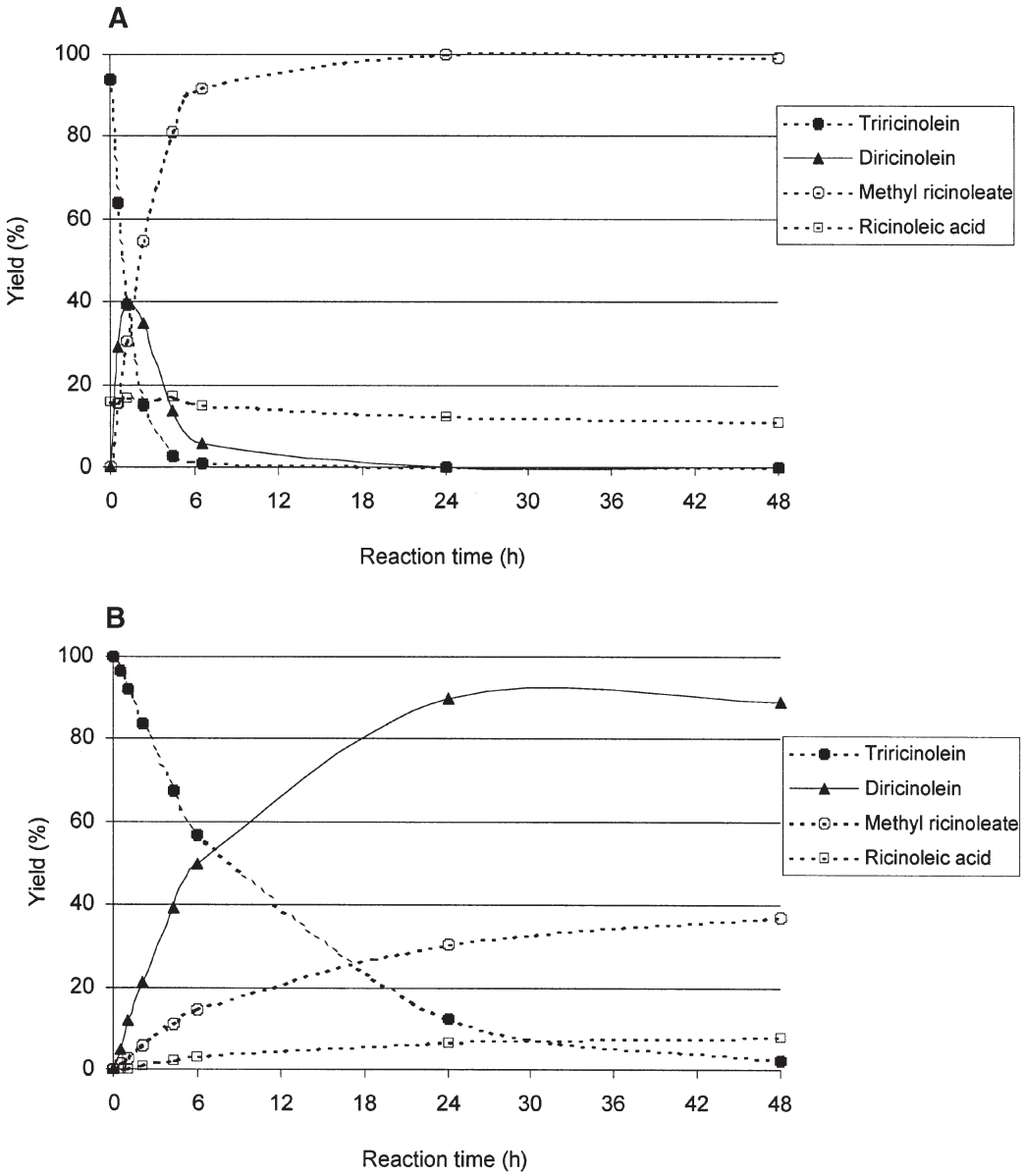

FIG. 5. Yield of diricinolein vs. reaction time for methanolysis of triricinolein in DIPE at an a_w of 0.11 catalyzed by (A) *Candida antarctica* type B lipase (CALB) and (B) PRL (n = 2). For abbreviations see Figure 2.

TABLE 1
Recoveries of Diricinolein (%) at Each Different Reaction Condition Tested (n = 2)[a]

Immobilized	a_w = 0.11		a_w = 0.53		a_w = 0.97	
lipase	Hexane	DIPE	Hexane	DIPE	Hexane	DIPE
CALB	40 (1 h)	40 (1 h)	16 (4 h)	26 (6 h)	20 (6 h)	17 (4 h)
RML	23 (1 h)	21 (1 h)	14 (1 h)	37 (1 h)	10 (48 h)	36 (1 h)
PCL	43 (7 h)	33 (4 h)	37 (1 h)	36 (4 h)	21 (1 h)	40 (4 h)
PRL	83 (48 h)	93 (24 h)	63 (48 h)	88 (48 h)	29 (48 h)	49 (48 h)

[a]The reaction times giving these maximum recoveries are shown within parentheses. a_w, water activity; CALB, *Candida antarctica* type B lipase; RML, *Rhizomucor miehei* lipase; PCL, *Pseudomonas cepacia* lipase; PRL, *Penicillium roquefortii* lipase; DIPE, diisopropyl ether.

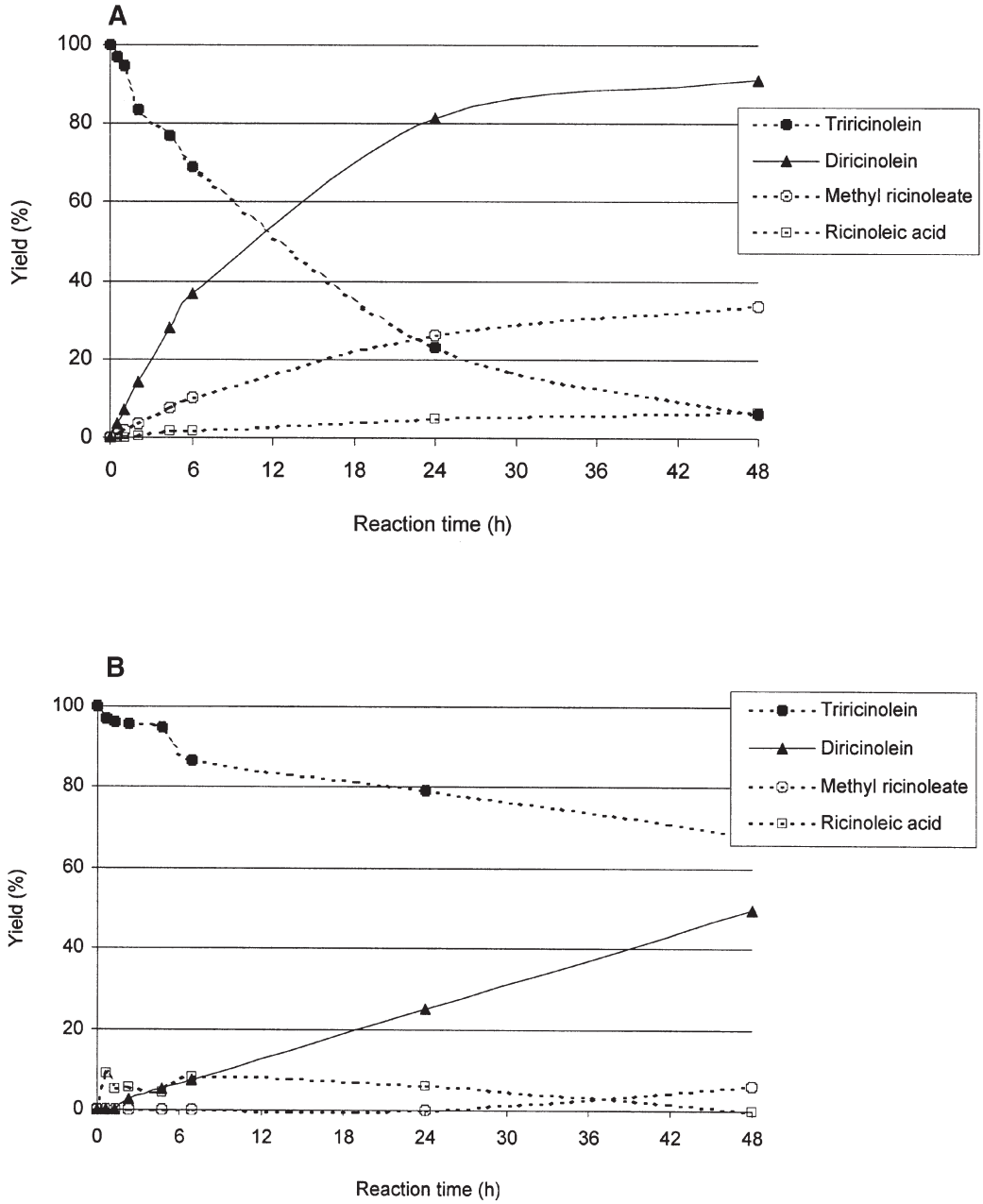

FIG. 6. Yield of diricinolein vs. reaction time for PRL-catalyzed methanolysis of triricinolein in DIPE at an a_w of (A) 0.53 and (B) 0.97 ($n = 2$). For abbreviations see Figure 2.

TAG (RRO) increased from 0 in the absence of diricinolein to 290 pmol/min/mg protein in the presence of 1 mM diricinolein. RRO was not produced in wild yeast cells when the same amount of 1,2(2,3)-diricinolein was supplied (data not shown). These results indicate that the diricinolein generated by lipase-catalyzed methanolysis is suitable as a substrate for the *Arabidopsis* DGAT, proving that diricinolein is suitable as a DGAT substrate.

In conclusion, PRL was chosen as the most suitable catalyst for the reaction, and DIPE was considered to be the better solvent, at an a_w of 0.53. The optimal reaction time was 24 h. These conditions gave a diricinolein yield of 88% and a purity of the 1,2(2,3)-isomer of 88%.

ACKNOWLEDGMENTS

We would like to thank USDA-IFAFS (the Initiative for Future Agriculture Food Systems) for Grant no. 2000-04820, which provided support for Charlotta Turner. We also gratefully acknowledge support for Xiaohua He from Dow Chemical Company through CRADA (Cooperative Research and Development Agreement) 58-3K95-2-918. We are grateful to Novozymes A/S for generously providing the Lipozyme RM-IM and Novozyme 435 enzymes, and Amano Enzymes Inc. for providing crude Lipase PS and Lipase R_G.

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[Received September 2, 2003, and in revised form October 13, 2003; revision accepted October 15, 2003]